# Evaluation of a Competitor Active in the Inhibition of Calcium Oxalate Crystal Growth Utilizing a Spectrophotometric Method

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## Objective:

A competitor ingredient will be evaluated for its ability to achieve inhibition in the *in vitro* growth of calcium oxalate crystals. The results will be compared to the patented formulation. This objective is divided into 2 goals:

1. Demonstrate the growth of calcium oxalate crystals in aqueous solution by UV-Visible spectroscopy.
2. Quantify the potency of the competitor ingredient for its activity in the inhibition of the growth of calcium oxalate crystals in aqueous solution.

## Summary:

The two objectives of this study were achieved. It was demonstrated by a spectrophotometric method that (1) calcium oxalate crystals could be grown in aqueous solution, and (2) the effect of the competitor ingredient on the % inhibition of crystal growth of calcium oxalate was determined.

## Materials:

All reagents were chemical grade or plant extracts.

| Reagent | Chemical Grade | Commercial Source | Lot # |
|---|---|---|---|
| Calcium chloride, dihydrate | ACS analytical grade | Mallinkrodt | G23H14 |
| Sodium oxalate | ACS Reagent ≥99.5% | Sigma-Aldrich | MKBP6508V |
| Sodium acetate | AR analytical reagent | Mallinkrodt | 7372KVND |
| Sodium Chloride | ACS Reagent ≥99.0% | Sigma-Aldrich | 040M0232V |
| Chanca Piedra | Herbal Supplement | Blue OrganiX | 101703 |

## Experimental Methods

## Spectrophotometric Method

The spectrophotometric method employed in this study was that based on the work of Chow (Chow, 2004) and is briefly described below. (For further background information on this method, refer to the Phase I & Phase II reports.) Based on the improvements in reproducibility that were observed in the Phase I and Phase II studies, the Phase VIII study was conducted using a sodium acetate/sodium chloride buffer at pH 5.71 as recommended by Khan (Kahn, 2012). The spectrometer employed was a Vernier SpectroVis spectrophotometer. Measurements were made in the Absorbance versus Time mode, monitoring at 620.6 nm, using ten (10) minutes for a full run with an acquisition rate of twenty (20) samples per minute.

The kinetics of calcium oxalate crystal formation are characterized by the slope method of Hess (Hess, 2000). Briefly, 1.6 milliliters of an 8.5 mM solution of calcium chloride is added to a plastic cuvette and 1.6 milliliters of a 1.5 mM sodium oxalate solution is added. Both of the solutions are prepared in a buffer composed of 50 mM sodium acetate and 100 mM sodium chloride at pH 5.71. Immediately upon combining the solutions, the cuvette is mixed by inversion and the kinetics of calcium oxalate crystal formation are monitored at 620.6 nm.

The kinetics of the inhibition of calcium oxalate formation are conducted using the method described in the previous paragraph with the following change. Before the sodium oxalate solution is added, 200 µL of the Test Solution of the competitor active is added to the cuvette. The remainder of the procedure is the same as described above. The inhibition of crystal growth is determined by comparison of the effect of the Test Solution on the slope of the initial velocity (compared to vehicle control, during the first two minutes), as described by Hess (Hess, 2000). Individual replicates identified as outliers by the Grubb's test are not included in the calculation (Grubbs, 1950).

The Test Solutions of the competitor active ingredient were prepared as described below (amounts given in Table

A1 in Appendix I).

**Matrix 1 & 2.** The Test Solutions for these two matrices were prepared by transferring the indicated amounts (as drops using the in-bottle provided dropper) of Chanca Piedra to a 100 mL volumetric flask and adding $dH_2O$ to bring the test solution to the final volume of 100 mL, vortexing to ensure complete dissolution. Matrix 1 included 40 drops of Chanca Piedra Extract 5:1 ratio for each run and Matrix 2 included 60 drops of Chanca Piedra Extract 5:1 ration for each run.

## Gravimetric Method

Three (3) gravimetric analyses were conducted to determine the amount of solid residue that was obtained on removal, via distillation, of the water and alcohol in which the herbal supplement was received. The process, described below, was used for all three samples. The contents of the herbal supplement bottle (dark brown, semi-viscous, sticky liquid) were poured into a pre-weighed 200- or 250-mL, one-necked round bottom flask. The bottle was rinsed with $dH_2O$ (2x) and 95% ethanol (2x). On the final rinse the liquid exiting the bottle was clear. The flask was placed on a rotary evaporator and the water/alcohol was removed at reduced pressure. The flask was then placed on a vacuum manifold and dried under vacuum for a period of forty eight (48) hours. The flask was removed from the vacuum manifold and weighed. The amount of insoluble residue was determined using mass by difference, see Table 2 in the Results & Discussion Section.

Based on the recommended usage, from the label on the herbal supplement bottle, a determination was made as to the volume, in mL, corresponding to three (3) different amounts of drops (again, using the in-bottle provided dropper). A determination was made for 20, 40, and 60 drops, with each determination run in triplicate. The process, described below, was used for all determinations. A 5 mL Pyrex graduated cylinder was used and the drops were placed directly into the graduated cylinder with the experimenter counting off the drops out loud. Once the desired amount of drops was added the graduated cylinder was visually inspected for the volume. See Table 2 in the Results & Discussion Section.

Another issue, from the label on the herbal supplement bottle, was the wording “one full dropper”. It was observed that when taking a sample directly from the bottle the glass dropper appeared to be only half-filled. The number of drops in this half-filled dropper was determined and this done twice to approximate what “one full dropper” would be in terms of drops, and using the information in the previous paragraph, what volume (in mL) this would correspond to, see Table 2 in the Results & Discussion Section.

## Results & Discussion

Figure 1 shows a representative full kinetic curve for the Vehicle runs 1-9 used for Matrix 1. From the figure it can be seen that calcium oxalate crystals are indeed forming during the ten minutes of the run. Figure 2 is the analysis of the slopes for the initial two minutes of Vehicle runs 1-9. Figure 3 shows full kinetic curves for Matrix 1 test solutions runs 1-9. Figure 4 shows the slope test of Matrix 1 runs 1-9**.**

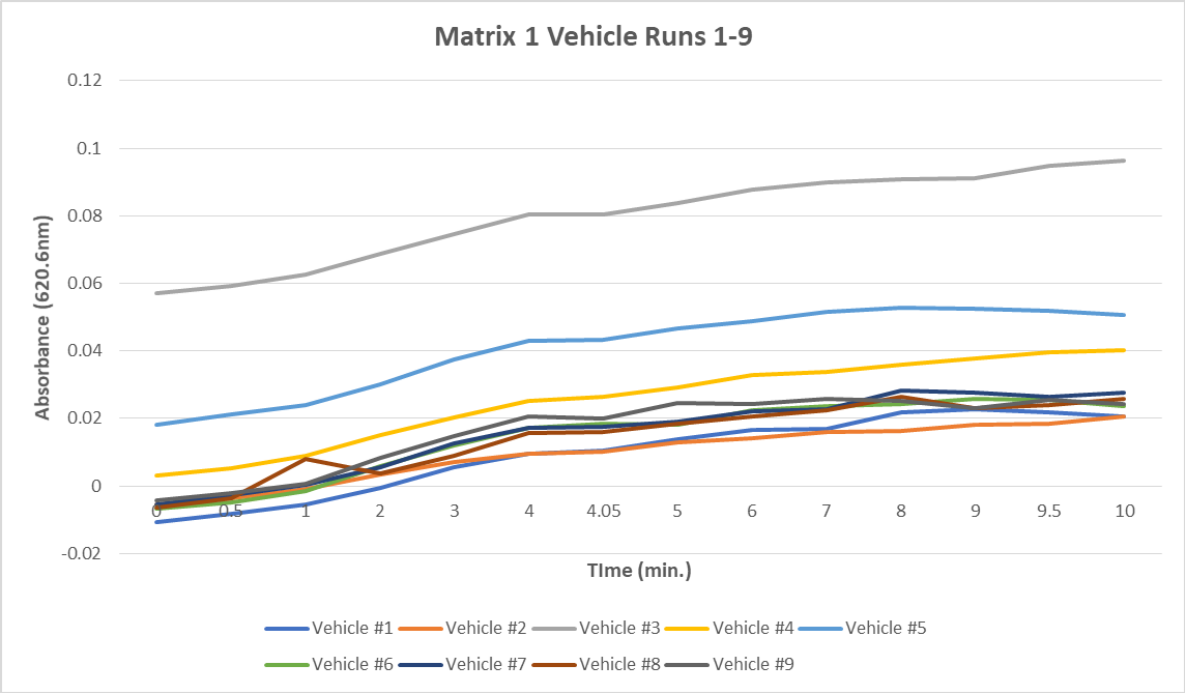


**Figure 1. Full Kinetic Curves for Vehicle Runs 1-9 Associated with Matrix 1**

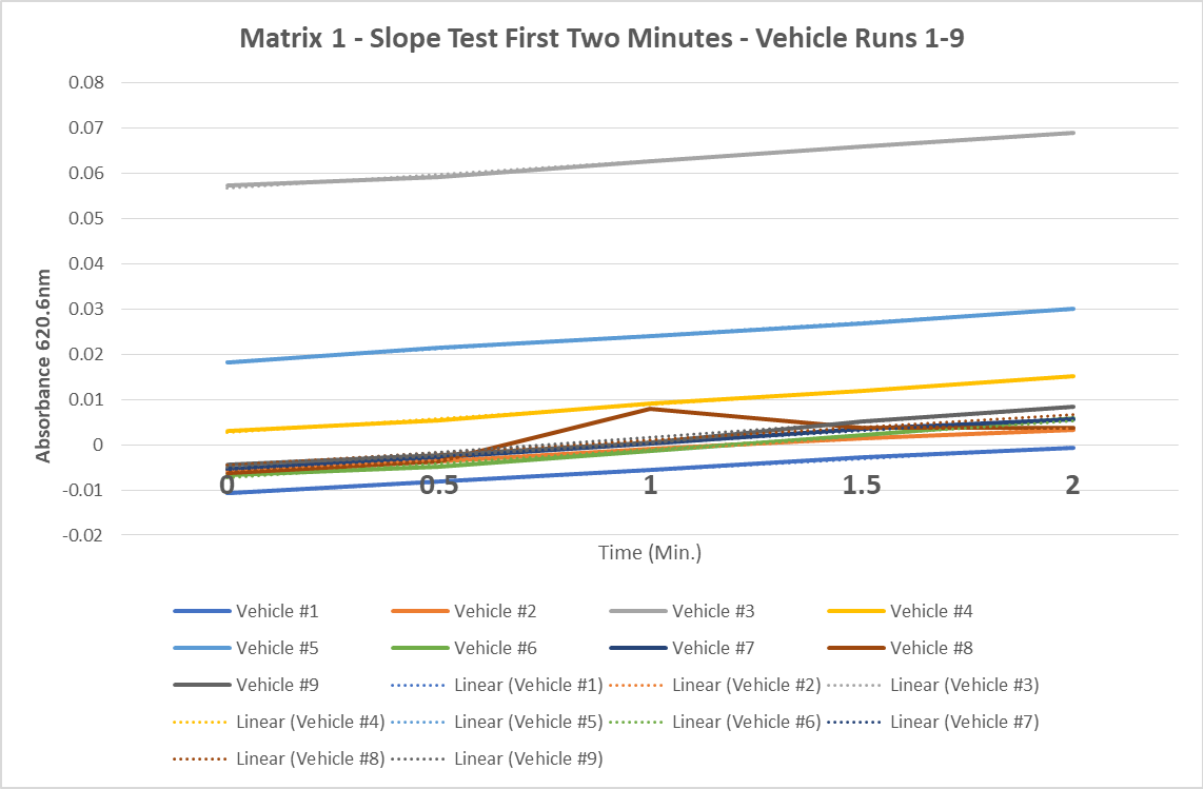


**Figure 2. Slope Test for Vehicle Runs 1-9 Associated with Matrix 1**

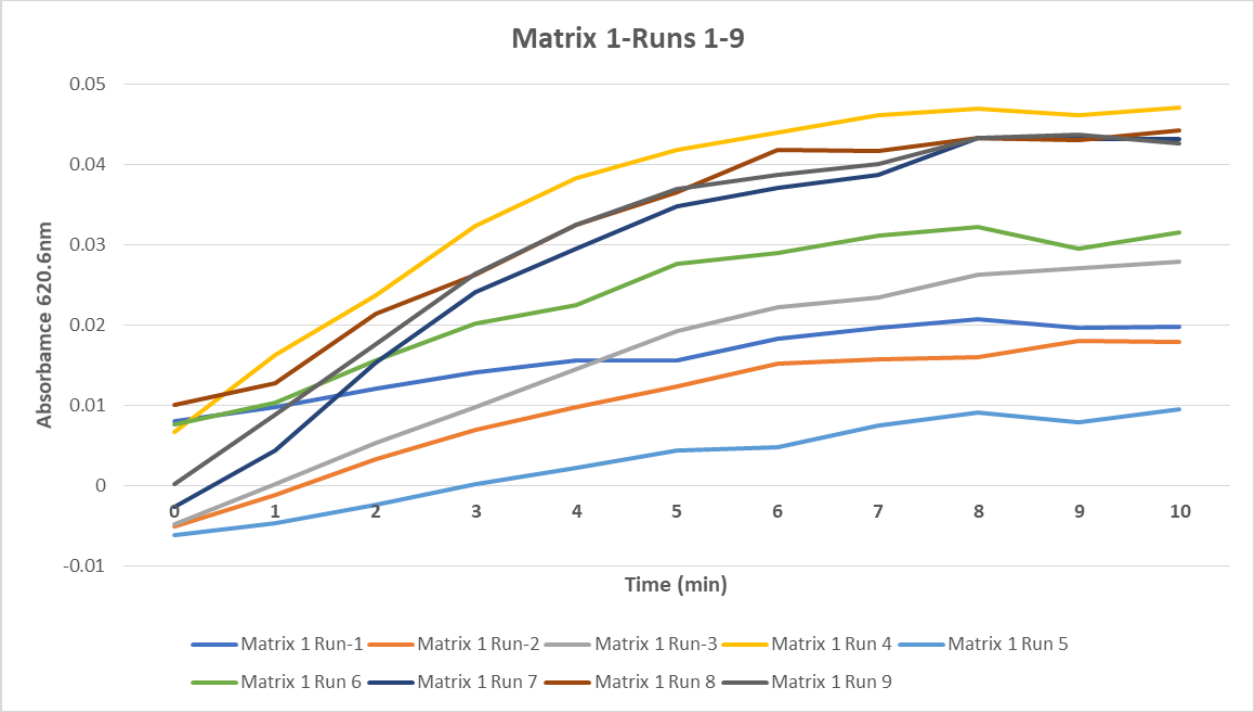


**Figure 3. Full Kinetic Curves for Matrix 1 Runs 1-9**

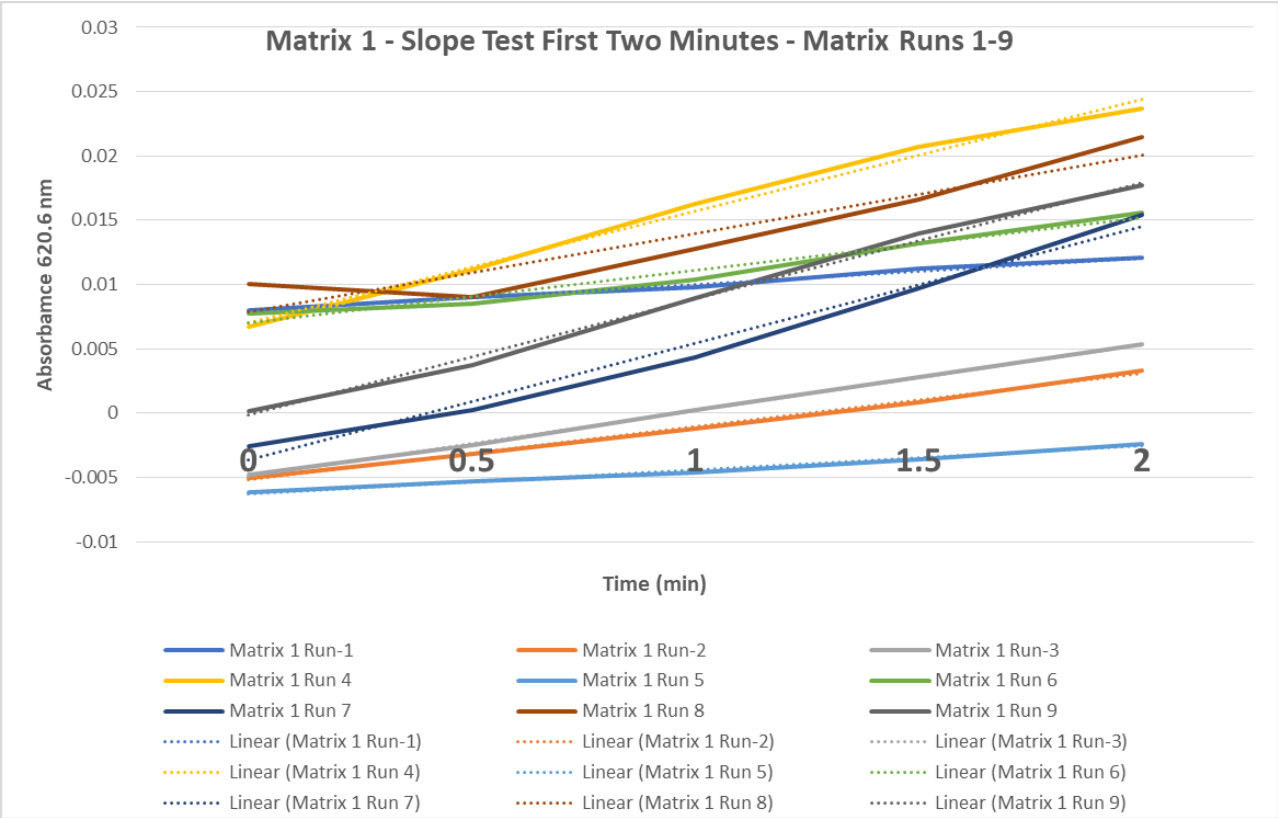


**Figure 4. Slope Test for Matrix 1 – Runs 1-9**

**Table 1. Data Analysis for Slope Test, Vehicle & Matrix 1**

| *Matrix 1* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Vehicle Run # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | *Median* |
| Slope | 0.0054 | 0.0045 | 0.0062 | 0.0065 | 0.0061 | 0.0066 | 0.0059 | 0.0053 | 0.0065 | *0.0061* |
| Matrix 1 Run # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| Slope | 0.0022 | 0.0039 | 0.0051 | 0.0092 | 0.0018 | 0.004 | 0.0093 | 0.0064 | 0.0092 | *0.0051* |
| Median % | 0.592592593 | 0.133333333 | 0.1774 | -0.41538 | 0.70492 | 0.39394 | -0.57627 | -0.20755 | -0.41538 | |
| | | | | | | | | | | |
| *% Median Inhibition* | **16%** | | | | | | | | | |
| *Average % Inhibition* | **4%** | | | | | | | | | |

**Table 2. Data Analysis for Slope Test, Vehicle & Matrix 2**

| *Matrix 2* | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Vehicle Run # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | *Median* |
| Slope | 0.009 | 0.0144 | 0.0095 | 0.0056 | 0.0172 | 0.0147 | 0.0044 | 0.006 | 0.0039 | *0.009* |
| Matrix Run # | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | |
| Slope | 0.0068 | 0.0085 | 0.0072 | 0.0073 | 0.0086 | 0.0101 | 0.0047 | 0.0027 | 0.0045 | *0.0072* |
| Median % | 0.244444444 | 0.409722222 | 0.2421 | -0.30357 | 0.5 | 0.31293 | -0.06818 | 0.55 | -0.15385 | |
| | | | | | | | | | | |
| *% Median Inhibition* | **20%** | | | | | | | | | |
| *Average % Inhibition* | **19.26%** | | | | | | | | | |

As an example of the data analysis, Table 1 shows the data used for determination of the slope, during the 0 to 2 minute time range, for Vehicle Runs 1-9 and for Matrix 1 Runs 1-9. Since the data was linear ($R^2$>0.95) for the first two (2) minutes of the reaction described above, this data was plotted for slope determination by linear regression analysis. Outlier data identified using the Grubbs' Test (Grubbs, 1950) were not included in % inhibition calculations. The % Inhibition was calculated according to the method of Hess (Hess, 2000) using the following equation:

% Inhibition = 1- (Slope of Test Mixture/Slope of Vehicle Solution)

From the results presented in Table 1 it is demonstrated that Matrix 1 did inhibit calcium oxalate formation at 16% inhibition. Using the same methodology, the results in Table 2 demonstrated that Matrix 2 inhibited calcium oxalate formation at 20% inhibition.

Table 3 presents the results of the various gravimetric analyses that were performed.

**Table 3. Data & Analysis for Gravimetric Analyses**

***Residual Material***

| | TS #1 | TS #2 | TS #3 |
|---|---|---|---|
| Final Mass | 98.26 | 114.53 | 115.28 |
| Initial Mass | 96.24 | 113.03 | 112.52 |
| *Difference* | *2.02* | *1.50* | *2.76* |
| ***Average*** | ***2.09*** | | |
| ***Median*** | ***2.02*** | | |
| ***Std. Dev.*** | ***0.63*** | | |

| | |
|---|---|
| ***2.092 g per 60 mL*** | ***34.87 mg per mL*** |

***Drops to mL***

| Drops out of Bottle | Run #1 mL | Run #2 mL | Run #3 mL | ***Average*** | ***Median*** | ***Std. Dev.*** |
|---|---|---|---|---|---|---|
| 20 | 0.60 | 0.60 | 0.60 | ***0.60*** | ***0.60*** | ***0.00*** |
| 40 | 1.00 | 1.00 | 1.00 | ***1.00*** | ***1.00*** | ***0.00*** |
| 60 | 1.40 | 1.40 | 1.40 | ***1.40*** | ***1.40*** | ***0.00*** |

Drops in "One Full Dropper"

| | 1st Half | 2nd Half |
|---|---|---|
| Test 1 | 40.00 | 39.00 |
| Test 2 | 39.00 | 39.00 |
| Test 3 | 39.00 | 38.00 |
| ***Average*** | ***39.33*** | ***38.67*** |
| ***Median*** | ***39.00*** | ***39.00*** |
| ***Std. Dev.*** | ***0.58*** | ***0.58*** |

**Conclusion**

The data indicate that the competitor active does inhibit calcium oxalate crystal growth, but only at approximately 16% inhibition for Matrix 1 (40 drops) and 20% for Matrix 2 (60 drops). With the determined % inhibitions being rather low, this can lead to scatter in the data from the UV/Vis assay. The preparation of the test solutions of the competitor inhibitor were simply done straight from the received bottle and the formulation was assumed from the ingredients listed on the label. The actual method of preparation of the plant extract is not known and there could be other material(s) present that could cause interference with the UV/Vis assay. In reviewing the results from the vehicle runs, the data appear very consistent, which indicates that the UV/Vis spectrophotometer is operating

properly and that the buffer and working solutions are good. If more information as to the preparation of the liquid herbal extract were known, this would allow for a more complete preparation of test solutions.

The results of the gravimetric analyses indicated that on average there were 2.09 grams of residual solid material for a 60 mL bottle of herbal extract. This translates to an average of 34.9 mg of residual solid material per 1 mL. From the drop comparison study (using the recommended 40-60 drops on the label) this would give an average range of 34.9 – 48.8 mg of solid residual material in one recommended dose. The instructions on the label indicate that this should be done three (3) times a day giving an average total daily range of 104.7 – 146.4 mg of residual solid material. In this analysis it is assumed that the residual solid material is all Chanca Piedra.

The results of the "one full dropper" study indicated an average of 39.3 drops for one half dropper and 38.7 drops for the second one half dropper. This gives an average total of 78 drops for "one full dropper". Using the number of drops/milliliters study (rounding to 80 drops) would give a little less than 2 mL of herbal extract, which would equate to a little less than 70 mg of residual solid for "one full dropper".

## Appendix

### Table A1 – Matrix 1 & 2 Ingredient Amounts

| **Matrix 1** | | |
|---|---|---|
| **Ingredient** | **Target (drops)** | **Actual (drops)** |
| **Chanca Piedra** | **40** | **40** |
| **Matrix 2** | | |
| **Ingredient** | **Target (drops)** | **Actual (drops)** |
| **Chanca Piedra** | **60** | **60** |

## References:


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3. Grubbs, Frank E., 1950, Sample criteria for testing outlying observations, *The Annals of Mathematical Statistics* 21(1), p.27-58.
4. Hess 2000, Citrate determines calcium oxalate crystallization kinetics and crystal morphology. *Nephrol Dialysis Transplant*, 15: 366-370.
5. Kalpana 2013, Inhibition of calcium oxalate crystallization in vitro by extract of banana cultivar monthan. *International Journal of Pharmaceutical Science*, 5(4): 649-653.
6. Khan 2012, Antiurolithic activity of *Origanum vulgare* is mediated through multiple pathways BMC *Complementary and Alternative Medicine* 11: 96-112.
7. Nakagawa 1981, Purification and Characterization of a Calcium Oxalate Monohydrate Crystal Growth Inhibitor from Human Kidney Tissue Culture Medium. *J. Biological Chemistry*, 256(8): 3939-3944.